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Synthesis of Aromatic $^{13}$C / $^2$H-$\alpha$-Ketoacid Precursors to be Used in Selective Phenylalanine and Tyrosine Protein Labelling†

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Recent progress in protein NMR spectroscopy revealed aromatic residues to be valuable information sources for performing structure and motion analysis of high molecular weight proteins. However, the applied NMR experiments require tailored isotope labelling patterns in order to regulate spin-relaxation pathways and optimize magnetization transfer. We introduced a methodology to use $\alpha$-ketoacids as metabolic amino acid precursors in cell-based overexpression of phenylalanine and/or tyrosine labelled proteins in a recent publication, which we have now developed further by providing synthetic routes to access the corresponding side-chain labelled precursors. The target compounds allow for selective introduction of $^{13}$C-$^2$H spin systems in a highly deuterated chemical environment and feature alternating $^{12}$C-$^{13}$C-$^{12}$C ring-patterns. The resulting isotope distribution is especially suited to render straightforward $^{13}$C spin relaxation experiments possible, which provide insight into the dynamic properties of the corresponding labelled proteins.

Introduction

Aromatic amino-acids represent a sensitive source of structural and dynamic parameters in high-molecular weight protein NMR spectroscopy.$^1$ Phenylalanines and tyrosines are substantially overrepresented at protein binding interfaces due to their ability to contribute to hydrophobic-, as well as to electrostatic interactions.$^2$ Examples from literature have proven the importance of aromatic residue derived NOE data to complement the set of methyl-group derived distance restraints for structure calculation.$^3$ Moreover, aromatic side chains display a remarkable flexibility in dynamic motion, which can be sensitively probed by $^{13}$C-$^1$H spin pair relaxation.$^4$ Insufficient chemical shift dispersion, extensive $^{13}$C-$^{13}$C spin coupling and retarded side-chain motion strongly effect the signal assignment and analysis in the aromatic spectral region.

Selective stable-isotope patterns are required to enable effective magnetisation transfer and well defined spin relaxation, which is both necessary to decrypt the structural information buried in these residues. Alternating $^{12}$C-$^{13}$C-$^{12}$C and/or $^2$H-$^2$H-$^2$H arrangements in the aromatic ring systems have been shown to result in well resolved NMR signals due to significant reduction of scalar and dipolar couplings.$^5$ Isolated $^{13}$C-$^1$H spin systems in an otherwise $^2$H-containing aromatic ring have additionally been used as valuable tools to elucidate aromatic side chain motion by erasing unwanted relaxation pathways.$^6$ Reports on labelling phenylalanine and tyrosine residues with stable isotopes include cell-free (CF) protein synthesis$^7$, as well as cell-based expression systems.$^6$ CF-approaches require the sophisticated synthesis of $^{15}$N-labelled amino acids, but display highly selective isotope composition in the target proteins. Cell-based overexpression, on the other hand, makes use of amino acid precursor compounds, which are introduced to the metabolism of a protein expressing organism.$^9$

Although economically preferred, cell-based methods often suffer from low incorporation rates and selectivity due to the loss of heavy isotopes at metabolic crossroads. In order to expand the methodology of introducing stable isotopes at distinct positions of a target protein, we recently presented highly selective phenylalanine- and tyrosine-residue labelling based on the corresponding metabolic $\alpha$-ketoacid precursors sodium phenylpyruvate and sodium 4-hydroxyphenylpyruvate (scheme 1).$^{10}$

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† Electronic Supplementary Information (ESI) available: $^1$H-NMR and $^{13}$C-NMR spectra of the target aromatic $\alpha$-ketoacid sodium salts 1-3 and the synthetic intermediates are provided. See DOI: 10.1039/b000000x/
Protein synthesis using an \textit{E. coli} overexpression host in the presence of the labelled aromatic \(\alpha\)-ketoacids thus resulted in the incorporation of \(^{13}\text{C}\) without any cross-labelling to other residues. This new methodology combines the robustness and versatility of in-cell overexpression with high incorporation selectivity, which is usually the domain of cell-free protein synthesis. In order to further develop our \(\alpha\)-ketoacid precursor based approaches towards selective side-chain labelling\(\textsuperscript{11}\), we developed a synthetic route to sodium phenylpyruvate \(\text{I}\) containing \(^{13}\text{C}-^{2}\text{H}\) at meta-positions in an otherwise perdeuterated chemical environment. We could already demonstrate that this side-chain labelled precursor is selectively converted to Phe-residues in an \textit{E. coli} expression medium.\textsuperscript{10} This article describes the synthetic details to obtain the \(^{13}\text{C}/^{2}\text{H}\) aromatic \(\alpha\)-ketoacids illustrated in scheme 2. In addition to the already mentioned precursor \(\text{I}\), synthetic approaches to access para \(^{13}\text{C}-^{2}\text{H}\) labelled phenylalanine precursor \(\text{2}\), as well as the meta \(^{13}\text{C}-^{2}\text{H}\) tyrosine precursor \(\text{3}\) are presented. The routes feature acetone and heavy water as \(^{13}\text{C}\) and \(^{2}\text{H}\) sources, respectively. Labelling of backbone positions is feasible by application of \(^{13}\text{C}\)-glycine as shown previously.\textsuperscript{10}

Results and discussion

The approach to access the target compounds \(\text{1-3}\) (scheme 2) is based on the synthesis of the aromatic ring by reaction of labelled acetone with nitromalonaldehyde in basic aqueous solution.\textsuperscript{12} Selective deuteration at activated ring-positions was planned in acidic \(\text{D}_{2}\text{O}\) using aniline or 4-aminophenol as electron rich substrates at elevated temperatures.\textsuperscript{13} On the one hand, this synthetic concept was designed as an economically practicable way of synthesizing enough material to be used in cell-based protein overexpression (quantitative isotope incorporation at 100-200 mg/L minimal medium) due to the relatively cheap sources of stable isotopes and robust reaction steps. On the other hand, the routes should be flexible enough to access alternative isotope patterns by simply switching to commercially available starting compounds with different stable isotope composition (e.g. various patterns of labelled acetone for side-chain-, or glycine as a \(^{13}\text{C}\)-source for backbone labelling).

The synthesis of sodium 3,3,3-dideuterio([3,5-\(^{13}\text{C}\)]2,4,6-trideuteriophenyl)pyruvate \(\text{1}\) was performed as outlined in scheme 3. Initially, a straightforward way to access the aromatic ring system in one step was applied by reaction of commercially available \([1,3-^{13}\text{C}]\) acetone \(\text{4}\) with sodium nitromalonaldehyde \(\text{5}\). Compound \(\text{5}\) can be prepared from muconic acid as a stable solid.\textsuperscript{14} Subsequent deoxygenation of \([2,6,^{13}\text{C}]\) 4-nitrophenol \(\text{6}\) was performed in a two-step reaction sequence via the 1-phenyl-\(1\text{-H}\)-tetrazoloylether \(\text{7}\).\textsuperscript{15} Compound \(\text{7}\) was prepared by reaction of the phenolic hydroxy group with 5-chloro-1-phenyl-\(1\text{-H}\)-tetrazole in the presence of KOtBu. Hydrogenation using palladium on charcoal at room temperature and a pressure of 4 bar removed the oxygen from the aromatic ring, while at the same time the nitro-group was reduced yielding \([3,5-^{13}\text{C}]\) aniline \(\text{8}\).\textsuperscript{16} At this stage, the deuterium pattern at the aromatic ring was installed, as compound \(\text{8}\) shows highly selective \(^{2}\text{H}/^{2}\text{H}\) exchange at the electron-rich ortho/para positions in presence of \(\text{D}_{2}\text{O}\) and HCl under microwave irradiation.\textsuperscript{15} Subsequent formation of \([3,5-^{13}\text{C}]\) 2,4,6-trideuteriobenzonitrile \(\text{10}\) was achieved by using potassium tetracyanonickelate in ammonium chloride buffer.\textsuperscript{17} Reduction of compound \(\text{10}\) using disopropylaluminium hydride yielded \([3,5-^{13}\text{C}]\) 2,4,6-trideuteriobenzaldehyde \(\text{11}\) which was then used in the subsequent condensation step with hydantoin.\textsuperscript{18} The preparation of labelled benzalhydantoin \(\text{12}\)
was done in the presence of ammonium acetate, which provided higher and more reproducible yields than the use of sodium acetate reported in literature.18a Finally, the hydantoin ring of compound 12 was hydrolysed using 20% NaOD solution, which simultaneously introduced 1H at the C9-position. Labelled sodium phenylpyruvate 1 was obtained by lyophilisation from aqueous solution as a stable white powder in an overall yield of ~16% in 8 steps from [1,3-13C] acetone 4.

![Scheme 3](image)

Scheme 3 Synthesis of labelled phenylpyruvate 1. Asterisks denote 13C labelling.

In order to access compounds 2 and 3, the deuteration of 4-aminophenol upon microwave irradiation was thoroughly studied (scheme 4). A nearly quantitative deuteration at position 3 and 5 was achieved within 30 minutes at 180°C in presence of D2O and HClconc. (1.25% v/v). Additional incorporation of 1H at position 2 and 4 was performed at a much slower rate with >95% deuteration after 8 hours and only minimal aminophenol degradation. The side-chain deuteration patterns for compounds 2 and 3 could thus be installed by varying the reaction time of the microwave mediated deuteration. Sodium 3,3-dideuterio[4-13C] 2,3,5,6-tetradideuterophenylpyruvate 2 was prepared by reducing [1-13C] nitrophenol 14 to [1-13C] aminophenol 15 using the continuous-flow hydrogenation reactor H-cube® (scheme 5). After microwave induced deuteration at positions 2,3,4 and 6, deoxygenation was again performed via the corresponding 1-phenyl-1H-tetrazolylether 17.

![Scheme 4](image)

Scheme 4 (A) Selective deuteration of 4-aminophenol. Reagents and conditions: 4-aminophenol (400 mg), D2O (4 ml), HCl conc. (50 µl), 180°C, microwave irradiation; (B) Time dependent progress of 4-aminophenol deuteration in positions 3,5 (A) and additional deuteration in positions 2,6 (B). The solvents were evaporated after 180 min. and replaced by fresh D2O. 1H content was analysed by integration of the corresponding 1H-NMR signals.

In this case, the Pd/C-mediated hydrogenation was again conducted in the continuous-flow hydrogenation reactor, leading to an isolated hydrogen atom in para position of the resulting labelled aniline 18. The following reaction steps were performed analogously to the reaction sequence reported for the preparation of the labelled sodium phenylpyruvate 1 leading to the target compound sodium 3,3-dideuterio[4-13C][2,3,5,6-tetradideutero-phenyl]pyruvate 2 in 9 steps and an overall yield of ~11%.

To achieve straightforward labelling at the aromatic side chain of tyrosine residues, a route to sodium 3,3-dideuterio[3,5-13C] 2,6-dideuterio-4-hydroxyphenylpyruvate 3 was developed as outlined in scheme 6. After formation of the aromatic system, [2,6-13C] 4-nitrophenol 6 was converted to [2,6-13C] 4-aminophenol 22 as described in the synthesis of compound 2. Deuteration in the ring positions 3 and 5 was then conducted in D2O / HCl at 180°C for 37 minutes, followed by formation of labelled 4-hydroxybenzonitrile 24 using K2Ni(CN)4. Disobutylaluminium hydride reduction gave 4-hydroxybenzaldehyde 25, which subsequently underwent condensation with hydantoin in the presence of piperidine. Hydrolysis of the hydantoin ring in NaOD / D2O finally gave sodium 3,3-dideuterio[3,5-13C] 2,6-dideuterio-4-hydroxyphenylpyruvate 3 as a stable white solid. This 7 steps sequence yielded the target compound 3 in a total yield of ~28%, which contains ~23% deuterium in positions 3 and 5 of the aromatic ring (determined by NMR signal integration).
A more selective deuteration pattern can be achieved, if required, as shown in scheme 7. Methylation of 4-nitrophenol and subsequent reduction of the nitro group yielded p-anisidine 28, which showed no reactivity in the deuteration step meta to the amino group (28 → 29). Demethylation using HBr in presence of a phase transfer catalyst (Aliquat-336®) gave selectively deuterated aminophenol 30. This sequence, which was verified using unlabelled 4-nitrophenol as a starting material, increases the number of reactions in the route to prepare sodium 3,3-dideuterio[1,5,3-D2,6-dideuterio-4-hydroxyphenyl] pyruvate 3 by two steps, but represents an effective approach to avoid partial deuteration at the 13C labelled aromatic positions in the target compound 3. The aromatic α-ketoacids 1-3 display high stability in their lyophilized forms as sodium salts, but undergo oxidative degradation in basic solution in presence of atmospheric oxygen. 1H NMR spectra of compound 1-3 in D2O show mainly the keto forms, whereas in DMSO-6d the enol forms predominate, which is in accordance with literature data.

Conclusions

An efficient synthetic concept is presented to access labelled metabolic precursor compounds of phenylalanine and tyrosine based on the low-cost isotope sources 13C-acetone and D2O. The routes enable the construction of specific labelling patterns in the aromatic side chains with special focus on alternating 12C-13C, 13C-12C ring sequences and isolated 13C-1H spin systems in an otherwise deuterated chemical surrounding. Highly selective aromatic side-chain labelling is thus feasible in cell-based overexpression systems without the need of chiral labelled amino acid additives. The resulting isotope arrangements facilitate the interpretation of Carr-Purcell-Meiboom-Gill (CPMG) based spin-relaxation experiments, improve the...
quality of aromatic proton NOE derived distance restraints and enable the unambiguous assignment of aromatic ring signals even in very large proteins. The precursors presented constitute valuable reporters of motional dynamics in complex molecular processes, such as protein folding, allostery and enzymatic catalysis. The straightforward and economic synthetic protocols shown will further promote the efforts to turn aromatic residue labelling into a routinely used concept and complement the techniques of NMR-based analysis of protein dynamics, which traditionally rely on the interpretation of spin relaxation residing at the backbone or 13C and 2H methyl bearing side-chains.24

Experimental section

General methods

All solvents were distilled prior to use. Anhydrous tetrahydrofuran and dimethylformamide were purchased from commercial suppliers. Dichloromethane was dried by elution over an aluminium oxide column. Isotope labelled reagents were purchased from Sigma-Aldrich ISOTEC with the following purity grades: [1,3-13C3] acetone (99% 13C), [2-13C] acetone (99% 13C) and D2O (99.9% 2H). Column chromatography was performed using silica gel 60 (0.040-0.063 μm, 240-400 mesh) from Merck. Thin layer chromatography (TLC) was done on precoated silica gel (Merck 60 F254) glass plates. TLC detection was carried out using a UVAC-60 neonlab ultraviolet lamp, an iodine chamber, or by application of a Mo-Ce(SO4)2 complex solution (48 g (NH4)2MoO4.4 H2O and 2 g Ce(SO4)2) in 100 mL 10% H2SO4). NMR spectra were recorded on a Bruker AVANCE-DPX 400 spectrometer at 400 MHz. Chemical shifts are given in parts per million (ppm). NMR solvent signals have been calibrated to the following ppm values: 2.5 (DMSO-d6), 4.79 (D2O), 7.26 (CDCl3) and 3.31 (CD3OD). NMR signal multiplicity is abbreviated as singlet (s), doublet (d), multiplet (m), doublet of doublets (dd), doublet of triplets (dt) etc. Mass spectrometry (MS) and high resolution mass spectrometry (HRMS) experiments were done using electron ionization (EI, 70 eV) or electrospray ionization (ESI, 3 keV). Continuous-flow hydrogelenations were performed in an H-Cube reactor from ThalesNano. Microwave reactions were conducted in a Biotage Initiator microwave synthesizer.

Sodium nitromalonaldehyde monohydrate 5: Sodium nitrite (30 g) was dissolved in water (30 mL) using a three necked round bottomed flask, equipped with a thermometer, a dropping funnel and a tube to drain the evolved gases. The mixture was slightly warmed to dissolve all of the NaNO2. A solution of mucobromic acid (30 g) in ethanol (30 mL) was slowly added during a period of 1 h. After additional stirring for 15 minutes, the reaction mixture was cooled to 0°C and the precipitate filtered off. The resulting solid was transferred into a round bottomed flask and stirred under reflux with ethanol (50 mL) and water (10 mL). The hot solution was filtered and the filtrate subsequently cooled to 0°C, which led to product precipitation. The solid was filtered off and washed with small portions of cold ethanol. Drying of the product in vacuum gave 7.67 g (42%) of sodium nitromalonaldehyde monohydrate 5 as a white solid, which was stored over CaCl2. 1H NMR (400 MHz, DMSO-d6): 9.72 (s, 2H, CHO); 13C NMR (DMSO-d6): 181.20 (CHO), 132.38 (C).

[2,6-13C2] 4-Nitrophenol 6: An aqueous NaOH solution (4.4 g in 20 mL) was slowly added to a mixture of sodium nitromalonaldehyde monohydrate 5 (3.25 g) and [1,3-13C3] acetone 4 (1 g) in H2O (200 mL) at 0°C using a dropping funnel. After the addition was completed, the flask was tightly closed and stirred for 6 days at 4°C. The resulting brown solution was cooled to 0°C and 6 N HCl (26 mL) was slowly added. Filtration of the solution resulted in a dark solid, which was taken up in 6 N HCl (26 mL) and boiled gently for 10 minutes. The warm mixture was filtered and the two combined filtrates were extracted with diethyl ether (6 x 100 mL). Subsequent drying of the combined organic phases over MgSO4 and evaporation of the diethyl ether under reduced pressure yielded a yellow solid. The crude product was purified over a silica gel chromatography column by elution with hexane / ethyl acetate (6:4 v/v). The reaction yielded 1.47 g (63%) of sodium nitromalonaldehyde monohydrate 5. Microwave reactions were conducted in a H-Cube reactor from ThalesNano. Microwave reactions were conducted in a H-Cube reactor from ThalesNano. Microwave reactions were conducted in a H-Cube reactor from ThalesNano. Microwave reactions were conducted in a H-Cube reactor from ThalesNano.
[3,5,13C3]2,4,6-Deuterioaniline 9: A microwave vessel (0.5-2 mL) was charged with [3,5,13C3] aniline 8 (338 mg), D2O (1.5 mL) and 10 drops of HCl conc. After the vessel had been irradiated for 10 minutes (150°C), the solvents were evaporated and the residue was again dissolved in D2O (1.5 mL). The vessel was tightly closed and again irradiated for 10 minutes (150°C). The procedure of evaporation, addition of D2O (1.5 mL) and application of microwave irradiation was performed two more times. The solution was then brought to neutral pH by addition of 1N NaOH and the product extracted with diethyl ether (3 x 60 mL). Drying of the organic phases over MgSO4 and subsequent careful evaporation of the solvents under reduced pressure (>100 mbar) gave 394 mg of a product / dichloromethane mixture which was used for further conversion. The reaction yield was determined by integrating the corresponding NMR signals to be 338 mg (97 %). 1H NMR (400 MHz, CDCl3): 7.16 (dd, J = 8.4 Hz, J = 7.4 Hz, J = 156.7 Hz, 2H, 13CH4), 6.80-6.62 (m, 3H, 13CH3), 3.63 (bs, 2H, NH2); 13C NMR (100.6 MHz, CDCl3): 129.69 (13CH); MS (EI): calcd. for C13H13N[M] 193.06; found 194.9.

[3,5,13C3]2,4,6-Trideuterioaniline 9: A solution of sodium nitrite (380 mg) in water (25 mL) was slowly added to a stirred mixture of [3,5,13C2]2,4,6-trideuterioaniline 9 (420 mg) in HCl (0.4%, 160 mL) at 0°C using a dropping funnel. After 2 h of stirring at 0°C, the reaction mixture was brought to pH 7 by addition of saturated aqueous Na2CO3. The resulting solution was slowly added to potassium tetracyanonioclate hydrate (1.06 g) in NH3 / NH4Cl buffer (60 mL, pH = 10). Stirring was continued for 15 min. at 60°C. The solution was then filtered and the solid residue washed with small aliquots of water. The combined filtrates were extracted with diethyl ether (4 x 100 mL) and the combined organic phases dried over MgSO4. Evaporation of the solvents under reduced pressure gave a crude product, which was further purified by bulb-to-bulb distillation (30 mbar; up to 120°C) to yield [3,5,13C3]2,4,6-trideuterioaniline 10 (387 mg, 84%) as a slightly yellow liquid. 1H NMR (400 MHz, CDCl3): 7.47 (dd, J = 8.1 Hz, J = 164.0 Hz, 2H, 13CH4); 13C NMR (100.6 MHz, CDCl3): 129.29 (13C); MS (EI): calcd. for C13H13D2N[M] 108.07; found 108.0.

[3,5,13C3]2,4,6-Trideuteriobenzaldehyde 11: A solution of [3,5,13C3]2,4,6-trideuterioaniline 10 (380 mg) in dry dichloromethane (30 mL) was set under argon atmosphere and cooled to -78°C. After the addition of disobutylaluminium hydride (1M in dichloromethane, 3.9 mL) was accomplished using a syringe, the mixture was allowed to 1.6°C during a period of 2 h. The reaction was quenched by addition of silica gel (5.4 g) and water (3 mL) in small portions. Subsequently, the mixture was stirred at 0°C for 1 h. The solution was transferred into an Erlenmeyer flask and a spatula of K2CO3 was added. After drying over MgSO4, the solids were separated off by filtration and rinsed with ethyl acetate (150 mL). Evaporation of the organic solvents under reduced pressure gave a crude product, which was purified using bulb-to-bulb distillation (20 mbar; up to 110°C). The reaction yield yielded [3,5,13C3]2,4,6-trideuteriobenzaldehyde 11 (332 mg; 86 %) as a colorless liquid. 1H NMR (400 MHz, CDCl3): 10.04 (s, 1H, CHO), 7.54 (dd, J = 7.8 Hz, J = 162.5 Hz, 2H, 13CH4); 13C NMR (100.6 MHz, CDCl3): 129.17 (13C); MS (EI): calcd. for C13H12D2O[M] 111.07; found 111.0.

5-([3,5,13C3]2,4,6-Trideuteriobenzylidene)hydantoin 12: A solution of [3,5,13C3]2,4,6-trideuteriobenzaldehyde 11 (225 mg), hydantoin (300 mg) and ammonium acetate (226 mg) was stirred in acetic acid (0.7 mL) using a round bottomed flask, was loaded with 5-([3,5,13C3]2,4,6-trideuteriobenzylidene)hydantoin 12 (217 mg) and set under a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser. The resulting solution was slowly added to potassium tetracyanonioclate hydrate (1.06 g) in NH3 / NH4Cl buffer (60 mL, pH = 10). Stirring was continued for 15 min. at 60°C. The solution was then filtered and the solid residue washed with small aliquots of water. The combined filtrates were extracted with diethyl ether (4 x 100 mL) and the combined organic phases dried over MgSO4. Evaporation of the solvents under reduced pressure gave a crude product, which was further purified by bulb-to-bulb distillation (30 mbar; up to 120°C) to yield [3,5,13C3]2,4,6-trideuterioenzonitrile 10 (387 mg, 84%) as a slightly yellow liquid. 1H NMR (400 MHz, CDCl3): 7.47 (dd, J = 8.1 Hz, J = 164.0 Hz, 2H, 13CH4); 13C NMR (100.6 MHz, CDCl3): 129.29 (13C); MS (EI): calcd. for C13H13D2N[M] 108.07; found 108.0.

Sodium 3,3-dideuterio([3,5,13C3]2,4,6-trideuteriophenyl) pyruvate 1: A two necked round bottomed flask, equipped with a reflux condenser, was loaded with 5-([3,5,13C3]2,4,6-trideuteriobenzylidene)hydantoin 12 (217 mg) and set under a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser, was equipped with a reflux condenser.
argan atmosphere. Addition of NaOD in D₂O (20%, 6 mL, prepared by slow addition of Na to D₂O) was accomplished via syringe and the mixture stirred at 100°C for 5 hours. After allowing the mixture to reach room temperature, the solution was extracted with diethyl ether (2 x 20 mL). The aqueous phase was brought to pH < 1 by slow addition of HClconc at 0°C. This mixture was then extracted with diethyl ether (5 x 30 mL) and the combined organic phases were dried over MgSO₄. The solvent was removed in vacuo yielding a white solid. To this residue D₂O (30 mL) was added and the pH set to 7 by careful addition of 1M NaOH. Lyophilization overnight yielded sodium 3,3,3-dideutero[(1,1′,1″,1‴C₃)] 2,4,6-trideuteriophenyl) pyruvate I (176 mg, 82%) as a white powder. NMR analysis showed residual ¹H at C₁ (< 5%). ¹H NMR (400 MHz, D₂O): 7.45 (dd, J = 8.1 Hz, J = 160 Hz, 2H, ¹3CHatom), 4.12 (s, 0.06 H, residual CH₂); ¹³C NMR (100.6 MHz, D₂O): 129.05 (¹3C); HRMS (ESI): calcd. for C₁3H12D8O [M – Na]⁺ 170.0777; found 170.0781.

[1-¹³C] 4-Nitrophenol 14: The synthesis was performed according to the preparation of [2,6⁻¹³C₂] 4-nitrophenol 6 using [2-¹³C] acetone as reagent. Purification of the raw product was achieved using column chromatography eluting with hexane/ethyl acetate (6:4) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆): δ = 11.02 (s, 1 H), 8.12 (dd, J = 9.2 Hz, J = 9.2 Hz, 2H, ¹3CHatom), 6.93 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, ¹3CHatom); ¹³C NMR (100.6 MHz, DMSO-d₆): 164.37 (¹3C), 126.65 (m-CH₂), 116.24 (d, J = 63.0 Hz, 2H, ¹3CHatom); HRMS (ESI): calcd. for C₁5H₁₃N₂O [M + H]⁺ 259.1326; found 259.1326.

[1-¹³C] 4-Aminophenol 15: [1-¹³C] 4-Nitrophenol 14 (1.28 g) was dissolved in MeOH (90 mL). This solution was conducted over a Pd/C (10%) catalyst cartridge in a continuous-flow hydrogen reactor (H-cube® – Thalesnano) at a flow-rate of 1 mL/min and room temperature. The hydrogen generator was set to full hydrogen mode. Evaporation of methanol under reduced pressure gave [1-¹³C] 4-aminophenol 15 (986 mg, 98%). ¹H NMR (400 MHz, DMSO-d₆): 8.30 (d, J = 2.1 Hz, 2H, OH), 6.47 (dd, J = 8.8 Hz, J = 8.8 Hz, 2H, ¹3CHatom), 6.42 (dd, J = 8.8 Hz, J = 2.1 Hz, 2H, ¹3CHatom), 4.36 (s, 2H, NH₂); ¹³C NMR (100.6 MHz, DMSO-d₆): 164.38 (¹3C), 141.12 (d, J = 8.8 Hz, 2H, ¹3CHatom), 140.86 (d, J = 66.0 Hz, 2H, ¹3CHatom); HRMS (ESI): calcd. for C₁₃H₁₄N₂O [M + H]⁺ 213.0828; found 213.0828.

[1-¹³C] 2,3,5,6-Tetrahydro-4-aminophenol 16: [1-¹³C] 4-Aminophenol 15 (980 mg) was heated to 180°C, together with D₂O (10 mL) and HClconc (125 µL) using a microwave reactor. The microwave vessel was purged with argon before the reaction was started. After 2.5 h, the reaction mixture was allowed to cool to room temperature, transferred to a round bottomed flask and the solvents were evaporated in vacuo. After addition of fresh D₂O (10 mL), the mixture was again transferred to a microwave vessel and irradiation was continued for another 5.5 h at 180°C. The solvents were then removed in vacuo and the residual black solid purified over silica gel column chromatography using ethyl acetate as the eluent. The reaction yielded [1-¹³C] 2,3,5,6-tetrahydro-4-aminophenol 16 (766 mg, 75%) as a light brown solid. NMR spectroscopy revealed quantitative deuteration at positions 2 and 6 and a deuteration grade of >95% in position 3 and 5. ¹H NMR (400 MHz, DMSO-d₆): 8.29 (s, 1H, OH), 6.46 (d, J = 3.0 Hz, 0.07 H, residual ¹3CHatom), 4.34 (s, 2H, NH₂); ¹³C NMR (100.6 MHz, DMSO-d₆): 148.48 (¹3C), 140.86 (d, J = 8.9 Hz, 2H, ¹3CHatom), 115.63 (dt, J = 62.0 Hz, J = 26.2 Hz, 2H, ¹3CHatom), 115.29 (t, J = 23.0 Hz, 2H, ¹3CHatom); HRMS (ESI): calcd. for C₁₃H₁₄D₆NO [M + H]⁺ 279.1326; found 279.1326.
2,3,5,6-tetradeuteriophenyl)pyruvate. Purification of the crude product using bulb-to-bulb distillation gave [4-13C] 2,3,5,6-tetradeuteriophenyl)pyruvate 19 (390 mg, 75%) as a colourless liquid. 1H NMR (400 MHz, DMSO-d6): 6.99 (d, J = 7.5 Hz, 0.08 H, residual m-CH$_{arom}$); 6.47 (d, J = 159.8 Hz, 1H, 13CH); 4.96 (s, 2H); 13C NMR (100.6 Hz, DMSO-d$_6$): 116.23 (13C); HRMS (ESI) calcd. for C$_{15}$H$_{13}$D$_2$N [M + H]$^+$ 99.0941; found 99.0940.

[4-13C] 2,3,5,6-Tetradeuteriobenzonitrile 19: The synthesis was conducted similar to the conversion of compound 9 to [3,5-13C] 2,4,6-trideuteriobenzonitrile 10. Purification of the crude product using bulb-to-bulb distillation gave [4-13C] 2,3,5,6-tetradeuteriobenzonitrile 19 (390 mg, 75%) as a colourless liquid. 1H NMR (400 MHz, DMSO-d$_6$): 7.61 (d, J = 161.5 Hz, 1H, 13CH$_{arom}$); 7.47 (d, J = 7.8 Hz, 0.08 H, residual m-CH$_{arom}$); 13C NMR (100.6 MHz, DMSO-d$_6$): 132.53 (13C); HRMS (EI) calcd. for C$_{15}$H$_{13}$D$_2$N [M] 108.0770; found 108.0697.

[4-13C] 2,3,5,6-Tetradeuteriobenzaldehyde 20: Compound 20 was synthesized according to the procedure described for the preparation of [3,5-13C] 2,4,6-trideuteriobenzaldehyde 11. The reaction yielded [4-13C] 2,3,5,6-tetradeuteriobenzaldehyde 20 (290 mg, 85%) as a yellow liquid. 1H NMR (400 MHz, CDCl$_3$): 10.04 (s, 1H, CHO), 7.63 (d, J = 160.0 Hz, 1H, 13CH$_{arom}$), 7.54 (d, J = 7.5 Hz, 0.08 H, residual m-CH$_{arom}$); 13C NMR (100.6 MHz, CDCl$_3$): 134.22 (13C); MS (EI) calcd. for C$_{15}$H$_{13}$D$_2$O [M] 111.08; found 111.1.

5-[(4-13C)] 2,3,5,6-Tetradideuteriobenzylidene)hydratoin 21: Preparation of compound 21 was accomplished similar to the preparation of 5-[(3,5-13C)] 2,4,6-trideuteriobenzylidene)hydratoin 12 yielding 363 mg (70%) of the target compound as a slightly green solid. 1H NMR (400 MHz, DMSO-d$_6$): 11.12 (bs, 1H, NH), 10.59 (bs, 1H, NH), 7.33 (d, J = 160.5 Hz, 1H, 13CH$_{arom}$), 7.10 (d, J = 7.3 Hz, 0.1 H, residual m-CH$_{arom}$), 6.41 (s, 1H, CH); 13C NMR (100.6 MHz, DMSO-d$_6$): 166.12 (CO), 156.28 (CO), 128.57 (arom.); 13C NMR (100.6 MHz, CDCl$_3$): 132.53 (13C); HRMS (EI) calcd. for C$_{15}$H$_{13}$D$_2$N$_2$O$_2$ [M + H]$^+$ 194.0949; found 194.0938.

Sodium 3,3-dideutero[4-13C] 2,3,5,6-tetradideuteriophenyl pyruvate 2: The synthesis of compound 2 was performed according to the preparation of sodium 3,3-dideutero[3,5-13C] 2,4,6-trideuteriophenylpyruvate 1, but using 5-[4-13C] 2,3,5,6-tetradeuteriobenzylidene)hydratoin 12 (85 mg) as a substrate. The reaction yielded sodium 3,3-dideutero[4-13C] 2,3,5,6-tetradideuteriophenylpyruvate 2 (76 g, 89%) as a colourless lyophilisate. NMR analysis showed residual 1H at C$_3$ (≤ 6%). 1H NMR (400 MHz, D$_2$O): 7.40 (d, J = 160.9 Hz, 1H, 13CH$_{arom}$), 4.13 (s, 0.11 H, residual m-CH$_{arom}$); 13C NMR (100.6 MHz, DMSO-d$_6$): 127.48 (13C); HRMS (ESI) calcd. for C$_{16}$H$_{15}$D$_2$O$_2$ [M - Na]$^+$ 170.0805; found 170.0803.

[2,6-13C$_2$] 4-Aminophenol 22: The reaction was performed analogously to the synthesis of [1-13C] 4-aminophenol 15 using [2,6-13C$_2$] 4-nitrophenol 6 (1.4 g) as a substrate. The reaction yielded [2,6-13C$_2$] 4-aminophenol 22 (985 mg, 90%) as a brown solid. 1H NMR (400 MHz, DMSO-d$_6$): 8.29 (t, J = 4.1 Hz, 1H, OH), 6.45 (dm, J = 153.2 Hz, 2H, 13CH$_{arom}$), 6.41 (d, J = 8.6 Hz, 2H, CH$_{arom}$), 4.35 (s, 2H, NH$_2$); 13C NMR (100.6 MHz, DMSO-d$_6$): 116.00 (13C); HRMS (ESI) calcd. for C$_{16}$H$_{15}$D$_2$NO [M + H]$^+$ 112.0673; found 112.0668.
separated off by filtration and rinsed with dichloromethane until no more product was washed out of the silica gel / MgSO₄ mixture (control of TLC spots under UV light). Evaporation of the combined organic phases under reduced pressure gave 504 mg (90%) of [3,5-13C₂] 2,6-dideuteriophenylglyoxaldehyde 25 as a yellow solid. 1H NMR (400 MHz, DMSO-d₆): 10.57 (s, 1H, OH), 9.79 (s, 1H, CHO), 7.78-7.73 (m, 0.15 H, residual o-CH₃(aro)); 13C NMR (100.6 MHz, DMSO-d₆): 115.69 (13C); HRMS (EI): calcd. for C₇₁₃C₂H₄D₂O₃ [M - Na]⁺ 208.0716; found 208.0716.

5-[(3,5,13C₂)[2,6-Dideutero-4-hydroxybenzyldiene] hydantoin 26: The reagents [3,5-13C₂] 2,6-dideuteriophenylglyoxaldehyde 25 (474 mg), hydantoin (423 mg) and piperidine (575 mg) were stirred in a 10 mL round bottomed flask, equipped with a reflux condenser at 130°C for 30 min. Addition of warm water (8 mL) was followed by homogenization of the resulting mixture in an ultrasonication bath. Precipitation of a solid was induced by adding HCl conc (0.5 mL). The crude product was separated by filtration and recrystallized from methanol, yielding 5-[(3,5,13C₂) 2,6-dideutero-4-hydroxybenzyldiene] hydantoin 26 (742 mg, 74%) as a yellow solid. 1H NMR (400 MHz, DMSO-d₆): 11.09 (s, 1H, NH), 10.30 (s, 1H, NH), 9.83 (s, 1H, OH), 7.50-7.43 (m, 0.12 H, residual CH(aro)); 6.78 (dm, J = 159.2 Hz, 1.47 H, 1H(aro)); 6.35 (s, 1H, CH); 13C NMR (100.6 Hz, DMSO-d₆): 116.07 (13C); HRMS (EI): calcd. for C₇₁₃C₂H₄D₂O₃ [M - Na]⁺ 208.0722; found 208.0716.

**Sodium 3,3-dideutero[3,5-13C₂] 2,6-dideutero-4-hydroxyphenyl pyruvate 3: A 10 mL round bottomed three-necked flask was charged with 5-[(3,5,13C₂) 2,6-dideutero-4-hydroxybenzyldiene] hydantoin 26 (50 mg) and set under argon atmosphere. A solution of NaOD in D₂O (20%, 4 mL) was degassed under argon by ultrasonication and added via syringe. Throughout the reaction a constant stream of argon was purged through the reaction mixture via a syringe needle to prevent oxidative degradation of the product. The mixture was stirred at 110°C for 4 h. After the reaction was allowed to cool to room temperature, the mixture was extracted with diethyl ether (2 x 20 mL). Subsequent addition of HCl conc (2.5 mL) to the aqueous phase was followed by extraction with diethyl ether (5 x 30 mL). The organic phases were combined and dried over MgSO₄. Evaporation of the solvents under reduced pressure gave a white solid to which D₂O was added (10 mL) and the resulting solution was brought to pH 7 by slow addition of NaOD (1N). Lyophilization yielded sodium 3,3-dideutério[3,5-13C₂] 2,6-dideutério-4-hydroxyphenylpyruvate 3 (44 mg, 88%) as a yellow solid. NMR analysis showed residual 1H at C(私下) (< 4%). 1H NMR (400 MHz, D₂O): 6.83 (dm, J = 163.9 Hz, 1.5 H, 1H(aro)); 3.99 (s, 0.07 H, residual CH(aro)); 13C NMR (100.6 Hz, D₂O): 115.69 (13C); HRMS (ESI): calcd. for C₇₁₃C₂H₄D₂O₃ [M - Na]⁺ 185.0663; found 185.0663.

3,5-Dideutero-4-aminophenol 30: 1.8-Diazabicyclo[5.4.0] undec-7-ene (DBU, 750 µL) was added to a stirred solution of 4-nitrophenol (700 mg) in acetonitrile (25 mL). The reaction mixture was stirred at room temperature for 10 min. before iodomethane (934 µL) was added drop-wise. After stirring the reaction mixture for 4 h, TLC still showed remaining starting material. Therefore, additional DBU (750 µL) and iodomethane (310 µL) were added and stirring continued for 1 h. The solvents were then removed under reduced pressure and the residue dissolved in ethyl acetate (100 mL). This solution was washed with 1 N HCl (10 mL), water (10 mL), a saturated solution of sodium thiosulfate (10 mL) and brine (10 mL). Drying of the organic phase over MgSO₄ and evaporation of the solvents yielded 4-nitroanisole 27 (686 mg, 90%). 1H NMR (400 MHz, CDCl₃): 8.21 (dm, J = 9.3 Hz, 2H, m-CH(aro)); 6.96 (dm, J = 9.3 Hz, 2H, o-CH(aro)); 3.91 (s, 3H, CH₃); 13C NMR (100.6 Hz, CDCl₃): 164.59 (C(aro)), 125.92 (CH(aro)); 114.02 (CH(aro)); 55.95 (CH₃). 4-nitroanisole 27 (546 mg) was dissolved in MeOH (40 mL) and conducted over a Pd/C (10%) catalyst cartridge in a continuous-flow hydrogen reactor (H-cube® – Thalesnano) at a flow-rate of 1 mL/min and room temperature. The hydrogen generator was set to full hydrogen mode. Evaporation of methanol under reduced pressure gave p-anisidine 28 (392 mg, 89%). 1H NMR (400 MHz, DMSO-d₆): 6.74 (dm, J = 8.9 Hz, 2H, m-CH(aro)); 6.65 (dm, J = 8.9 Hz, 2H, o-CH(aro)); 3.75 (s, 3H, CH₃), 4.41 (bs, 2H, NH₂); 13C NMR (100.6 Hz, DMSO-d₆): 152.87 (C(aro)), 139.94 (C(aro)), 116.42 (C(aro)), 114.85 (C(aro)), 55.76 (CH₃). A microwave vessel was charged with anisidine 28 (272 mg), D₂O (2.5 mL) and HCl conc (50 µL) and heated in the microwave reactor at 180°C for 40 min. After the solvents had been removed under reduced pressure, the residue was dissolved in methanol (10 mL) and concentrated again to yield 2,6-dideutério-p-anisidine 29 (245 mg, 90%). 1H NMR (400 MHz, DMSO-d₆): 6.73 (s, 2 H, CH(aro)); 6.37 (bs, 2H, NH₂), 3.65 (s, 3H, CH₃); 13C NMR (100.6 Hz, DMSO-d₆): 153.14 (C(aro)), 138.15 (C(aro)); 114.95 (CH(aro)), 55.81 (CH₃); HRMS (EI): C₇₁₃C₂H₄N₂O₃ 125.0804; found 125.0803. 2,6-Dideutério-p-anisidine 29 (96 mg) was treated with HBr (47%, 620 mL) and Alikat-336 (16 mg) at 105°C for 6 h. The reaction was quenched by addition of water (5 mL) and the resulting solution extracted with ethyl acetate (50 mL). After the aqueous phase was brought to pH > 12 by addition of 1M NaOH, the mixture was extracted with ethyl acetate (3 x 50 mL). The organic phases resulting from the second extraction were combined, washed with water (2x 20 mL) and dried over MgSO₄. The crude product was purified over a short silica-gel column using ethyl acetate as an eluent to yield 3,5-dideutério-4-aminophenol 30 (63 mg, 73%). 1H NMR (400 MHz, DMSO-d₆): 8.30 (s, 1H, OH), 6.46 (s, 2H, o-CH(aro)); 4.34 (s, 2H, NH₂); 13C NMR (100.6 Hz, DMSO-d₆): 148.16 (C(aro)), 140.52 (C(aro)), 115.07 (o-CH(aro)).

**Notes and references**


